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Synthesis and delivery activity of new cationic cholesteryl glucosides

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ABSTRACT

Cholesterol amphiphiles containing positively charged groups (pyridinium, *N*-methylimidazolium, *N*-methylmorpholinium, and *N*-methylpiperidinium) linked via β-glucosyl spacer were prepared by alkylation of the corresponding bases with 6-*O*-mesyl-β-D-cholesteryl glucopyranoside. IC_{50} values were in the range 20–35 μM for the series of compounds and liposomal formulations with DOPE (1:1) were significantly less toxic. The liposomal formulations provided the accumulation of FITC-labeled oligonucleotide in cells, and the efficiency of this process was comparable to that of Lipofectamine® 2000. Cationic liposomes were able to deliver siRNA into the cells, and the liposomal formulation **7d**/DOPE provided the most pronounced down-regulation of EGFP expression both in the presence and in the absence of serum (up to 30%).

1. Introduction

Gene therapy, as an alternative to the conventional medicine, implies eliminating the cause of a disease by introducing DNA or oligonucleotides (antisense oligonucleotide or siRNA). Before therapeutic nucleic acids reach the nucleus, they have to overcome a number of biological barriers, in particular, cell, endosomal, and nuclear membranes. This is attained by using appropriate delivery systems, protecting the genetic material from the destructive action of enzymes and promoting their penetration into the intracellular space, transfer through the nuclear membrane, and further expression in the nucleus.[1–3] In addition, these delivery systems should be nontoxic, nonimmunogenic and biocompatible.

Lipofection, a method based on the use of cationic liposomes for gene transfer, occupies an important place among the methods of gene delivery into eukaryotic cells.[4] To perform lipofection, it is necessary first to form cationic liposomes or micelles and then their complexes with plasmid DNA or oligonucleotides, called lipoplexes. Cationic liposomes are formed using cationic amphiphiles, comprising a wide range of chemical compounds with common structural features, namely, the presence of positively charged and hydrophobic domains separated by spacers of various lengths.[5–9] Biodegradable cationic amphiphiles of natural origin are of most interest now. Recently, glycerol- and cholesterol-containing cationic amphiphiles with various cationic 'heads' attached to the hydrophobic moiety via a spacer by means of a carbamoyl, ester, or ether bond have been synthesized.[5,7–9] Targeted modification of each domains gives rise to a series of amphiphiles, which can be used to study the structure–activity relationship. As a rule, it is possible to compare results only in experiments with the same cell line.

It is known that the efficiency of liposome-mediated gene delivery is determined not only by the structure of cationic and helper lipids, properties of the plasmid, but also by the size and ζ-potential of the lipoplex.[10] The structure of supramolecular DNA–lipid complexes depends on both external (pH, degree of hydration, temperature, and the presence of doubly charged cations, that is, Ca^{2+}, Mg^{2+})[6,11] and internal factors. The internal factors mean the phase state of the lipid molecule (lamellar or hexagonal), which is governed by the ratio of space areas of the polar and hydrophobic domains.[6,7,12,13] The incorporation of a helper lipid, such as dioleoyl phosphatidylethanolamine (DOPE), in liposomal formulations can significantly enhance the gene delivery activity of the cationic lipid. The zwitterionic phospholipid DOPE induces a strong destabilizing effect on lipid bilayers due to its spatial organization.[14,15] This results in a facilitated disruption of the endosomal membrane in transfected cells and subsequent cytosolic release of a delivered cargo. To increase the transfection level at the endosomal release stage, especially 'sensitive' elements irreversibly transformed by the action of cell medium have been introduced into the cationic lipid structure.[7,16–18] Therefore, the efficiency of lipid vehicles for the nucleic acid delivery finally depends on the structure of cationic amphiphile whose chemical design requires a tailored approach taking into account the lipid composition of membranes, the nature of cell receptors, and the chemical processes in the intracellular environment.

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Cationic amphiphiles containing cholesterol as a hydrophobic residue possess a high transfection activity and a low toxicity and find use in studies of both the structure–activity relationships and the membrane fusion mechanisms. Among synthesized cholesterol derivatives, there is a commercially available lipid, 3-β-[*N*-(*N*′,*N*′-dimethylaminoethyl)carbamoyl]-cholesterol (DC-Chol). Liposomes prepared with DC-Chol and DOPE lipids are widely used to deliver the plasmid DNA for tumor immunotherapy[19,20] or artificial immunization[21] and are under clinical trials for the therapy of mucoviscidosis.[22]

As mentioned above, lipids with different positively charged groups based on aliphatic or heterocyclic bases were synthesized to reveal the structure–activity relationships between the structure of cationic lipids and the transfection activities. It was found that heterocyclic cationic lipids- containing imidazolium[23] or pyridinium polar heads[24–27] displayed higher transfection efficiency and reduced cytotoxicity when compared to classical transfection systems. Recently, we performed systematic study on the synthesis of lipofection mediators with the hydrophobic component represented by diglycerides, cholesterol, and fatty acid residues. Heterocyclic bases pyridine, *N*-methylimidazole, and *N*-methylmorpholine were used for the creation of a cationic 'head' in diglycerides[28,29] and cholesterol.[30–32] Previously, we also synthesized a number of cationic carbohydrate-containing glycerolipids differing in the structure and the position of the positively charged group and the type of linker between the carbohydrate and the glycerol fragment.[33,34] To get further development in this direction, herein we describe the synthesis of novel cationic cholesteryl glucosides with different heterocyclic headgroups, which may serve as mediators of gene transfer into eukaryotic cells.

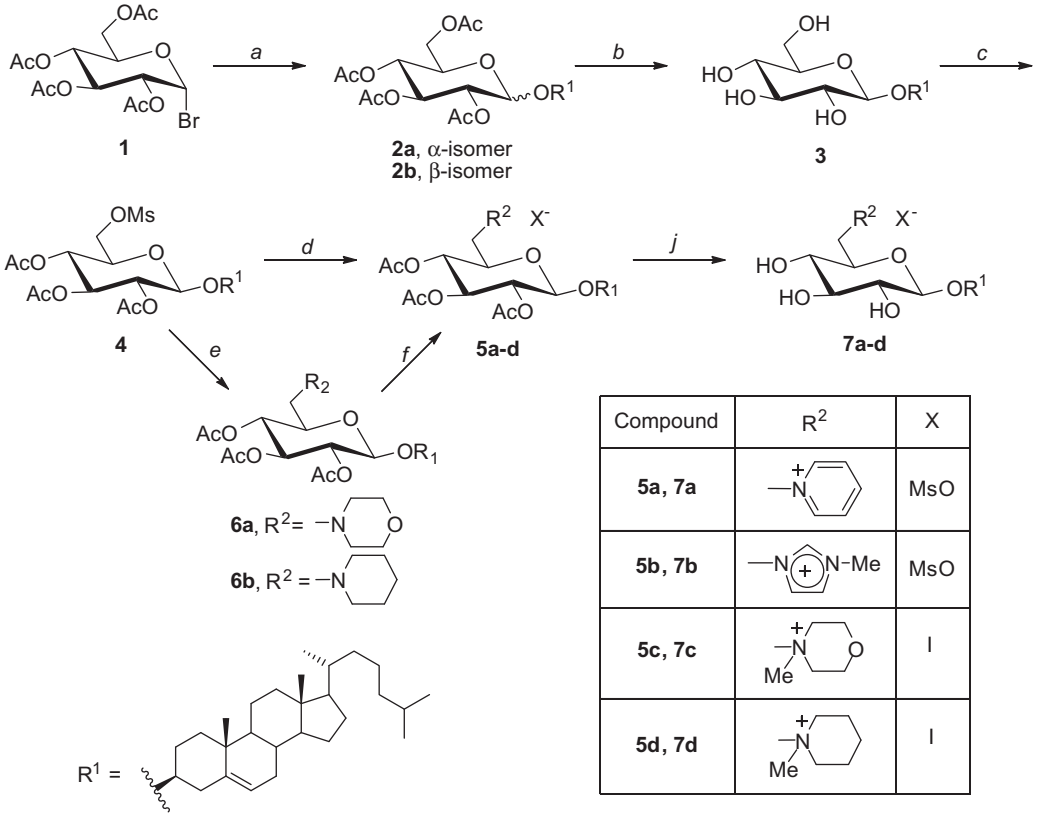
2. Results and discussion

2.1. Synthesis of cationic glucolipids

It is known that the presence of a carbohydrate residue increases the colloidal stability of DNA–cationic liposome complexes, enhances DNA delivery, and decreases the cytotoxicity of cationic amphiphiles.[6,35–37] Our synthetic strategy considers the glycosyl unit as a sort of biodegradable linker. According to the preliminary biological assays, the efficiency of transfection of eukaryotic cells using cationic glycerolipids increases when a carbohydrate spacer group is introduced into the structure.[38]

The goal of this work was to prepare cholesterol-containing amphiphiles **7a–d**, containing a β-glucosyl unit as a spacer, in which cationic groups are attached directly to C-6 of the carbohydrate residue (see Scheme 1). We used pyridinium, *N*-methylimidazolium, *N*-methylmorpholinium, and *N*-methylpiperidinium groups as cationic heads and employed these cationic heads in our previous study to obtain cationic glycerolipids.[28–32] Cholesterol was chosen as a hydrophobic component due to its biological compatibility and the ability to stabilize membranes and to form rigid liposomes.

The glucosides **2a,b** were obtained by condensation of acetobromoglucose **1** and cholesterol at a 1:2 molar ratio under Helferich reaction conditions[39] in toluene in the presence of $Hg(CN)_2$ and dried molecular sieves (4 Å) at 100 °C. This approach is widely used in steroid glycoside chemistry owing to a high promoting activity of mercury salts and the easy accessibility of the glycosyl donor.[40] The total yield and the stereoselectivity of this reaction largely depend on the nature and the structure of the steroid alcohol. The condensation gave two anomers with a total yield of 63%.



Compound	R^2	X
5a, 7a		MsO
5b, 7b		MsO
5c, 7c		I
5d, 7d		I

Scheme 1. Reagents and conditions: (a) cholesterol, $Hg(CN)_2$, 63% or $CdCO_3$, MePh, 52%; (b) MeONa/MeOH, 90%; (c) MsCl, then Ac_2O, Py, 70%; (d) Py or MeIm, 78% (80%); (e) morpholine or piperidine, then Ac_2O, 52% (58%); (f) MeI, 82% (72%); and (g) 0.04 N MeONa/MeOH.

Chromatographic separation and analysis of ^{1}H and ^{13}C NMR data suggested that α- and β-glucosides were formed in a ratio of 1:2.3. A sharp increase in the glycosylation stereoselectivity (α/β, 1:51) with a slight decrease in the total yield (52%) was attained by replacing $Hg(CN)_2$ with $CdCO_3$ and conducting the process in a Soxhlet apparatus for 3 h according to the known procedure.[41] β-Anomer **2b** was subsequently deacetylated by Zemplen reaction with 0.2 N sodium methoxide in methanol yielding cholesteryl β-D-glucoside (**3**) after recrystallization in 90% yield.

The mesyl group was used as a leaving group when introducing the cationic 'head' into the glucoside molecule. A regioselective mesylation at the carbohydrate C-6 atom of cholesteryl β-D-glucoside (**3**), which was accomplished by the treatment with an equimolar amount of mesyl chloride at −15 °C,[19] resulted in a complete disappearance of the starting glucoside **3** from the reaction mixture to give a series of new products containing different numbers of mesyl groups with predominance of the 6-*O*-mesyl derivative. For more convenient purification of the product and prevention of by-product formation during subsequent elimination of the mesyl group under basic conditions, acetylation with acetic anhydride and chromatographic separation were performed to give the major reaction product **4** in 70% yield.

Two approaches were used to introduce the cationic group into cholesteryl glucoside. In the first approach, direct quaternarization of heterocyclic bases with mesyl ester **4** was used. Heterocyclic bases were quaternarized either in the medium of the base itself (in the case of pyridine) or in the presence of an organic solvent, methyl ethyl ketone (in the case of other bases). The reactions of mesyl ester **4** with pyridine and *N*-methylimidazole proceeded for 9 and 19 h at 115 and 100 °C, respectively. After recrystallization, yields of compounds **5a** and **5b** were 78% and 80%, respectively. Quaternarization of *N*-methylmorpholine and *N*-methylpiperidine was unsuccessful and gave cationic lipids **5c,d** in low yields (<5–10%); therefore, another approach was proposed in order to obtain lipids **5c,d**. A two-stage route comprising alkylation of morpholine and piperidine with mesylate **4** followed by quaternarization with methyl iodide provided lipids **5c** and **5d** under milder conditions in good yields. Alkylation of cyclic amines was accompanied by partial deacetylation of the carbohydrate component due to a high basicity of used bases, which was confirmed by ^{1}H NMR data for fractions obtained upon chromatographic separation of the reaction products. Therefore, after alkylation of heterocyclic amines, the mixture of compounds with different numbers of acetyl groups was acetylated to give compounds **6a** and **6b** in 52% and 58% yields after chromatographic purification. At the next stage, tertiary amines **6a** and **6b** were quaternized with methyl iodide to give target cationic lipids **5c** and **5d** in 82% and 72% yields, respectively.

2D Homonuclear (COSY) and $^{1}H/^{13}C$ heteronuclear (HSQC) NMR spectroscopy techniques were used to assign signals from the glucosyl residue and to confirm structures of compounds **5a–d**. We found that in the case of compounds **5c** and **5d**, the signals for the anomeric proton (δ_{1H} 5.40–5.48 ppm) and the proton at C-5 (δ_{5H} 4.70–5.00 ppm) shifted downfield as compared with the signals of these protons for compounds **5a** and **5b**, which occur at high field (δ_{1H} 4.60–4.70 ppm, δ_{5H} 4.12–4.25 ppm). Presumably, the presence of a positively charged nitrogen atom near the pyranose ring in compounds **5c** and **5d** induces a decrease in the electron density on the ring oxygen atom. In turn, this results in the electron density redistribution on the neighboring C-1 and C-5 atoms, which is evident from the NMR spectrum. In the case of compounds **5a** and **5b**, the positive charge is delocalized through the aromatic ring π-orbitals; therefore, the nitrogen atom is less electrophilic and cannot interact with the pyranose oxygen atom.

Zemplen deacetylation of quaternarization products **5a–d** was the final stage in the synthesis of the cationic cholesteryl β-D-glucosides. Target cationic cholesteryl glucosides **7a–d** were isolated in 45–89% yields by reverse phase column chromatography on LiChroprep® RP-18.

2.2. Biological testing of cationic glycolipids

To assess the potential of the synthesized cationic glycolipids as new gene delivery agents, we estimated biological effects of these compounds on cell viability and cytosolic delivery of a 25-mer fluorescein-labeled oligodeoxyribonucleotide (FITC-ODN) and a small interfering RNA (siRNA). These biological tests were performed for both individual glycolipids and liposomes. The liposomal formulations were composed of one of the cationic glycolipids **7a–d** and the zwitterionic phospholipid DOPE at a molar ratio of 1:1. At this molar ratio, maximum levels of transfection were observed for a number of cationic lipids.[42,43] For the liposomes preparation, a thin lipid film was hydrated and sonicated to give the desired formulation.

2.2.1. Estimation of the cytotoxicity of cationic glycolipids 7a–d and liposomal formulations

The cytotoxicity of the cationic glycolipids **7a–d** was evaluated by the MTT assay.[44] In these experiments, BHK cells were incubated with various concentrations of cationic glycolipids **7a–d** or cationic liposomes composed of glycolipids and DOPE (molar ratio of 1:1) for 24 h and then treated with MTT. The toxic effect of cationic lipids (Fig. 1A) followed a typical S-shaped curve both in the absence and in the presence of serum in the cell medium. In the absence of serum, the IC_{50} values for lipids **7a–d** were equal or exceeded 25 μM, while a small enhancement in glycolipid cytotoxicity was observed in the presence of serum (Fig. 1A). The structure of the positively charged group in cationic lipids **7a–d** had no significant influence on the cell growth; however, lipid **7c** with the *N*-methylmorpholinium head was slightly less toxic as compared with the other cationic lipids.

An addition of DOPE to cationic glycolipids significantly reduced their cytotoxicity: IC_{50} values exceed 100 μM (Fig. 1B). It was observed that all liposomes stimulated cell proliferation at concentrations from 20 to 80 μM.

2.2.2. Cellular delivery of FITC-labeled oligonucleotides in the presence of cationic glycolipids

To estimate the ability of cationic glycolipids to mediate nucleic acid transfer across the plasmatic membrane, the delivery of FITC-ODN into BHK cells was studied by FACS analysis using lipid concentrations of 10 and 20 μM. The FITC-ODN concentrations were 0.8 μM and 5 μM corresponding to the range of concentrations at which a down-regulation of a gene expression can be observed and concentrations of glycolipids did not exceed the IC_{50} values of individual compounds. In the first screening study, complexes of glycolipids **7a–d** with FITC-ODN were formed at four different ODN/cationic lipid molar ratios (5:10, 5:20, 0.8:10, and 0.8:20) that correspond to N/P (nitrogen to phosphate) ratio of 0.08, 0.16, 0.5, and 1, respectively. Lipoplexes were preformed in OptiMem medium for 30 min at room temperature and added to BHK cells. After 4 h incubation with FITC-ODN/glycolipids complexes, the cells were fixed and analyzed. The percentage of the FITC-positive cells and the cell-associated fluorescence intensity was measured. The results showed that FITC-ODN delivery into BHK cells mediated by glycolipids **7a–d** was poor: no more than 8% of the BHK cell population was transfected with FITC-ODN. Interestingly, the cellular accumulation of FITC-ODN/glycolipid (20 μM) complexes was two to threefolds higher in the presence of serum as compared with serum-free conditions (5% and less than 1.5% of FITC positive cells, respectively).

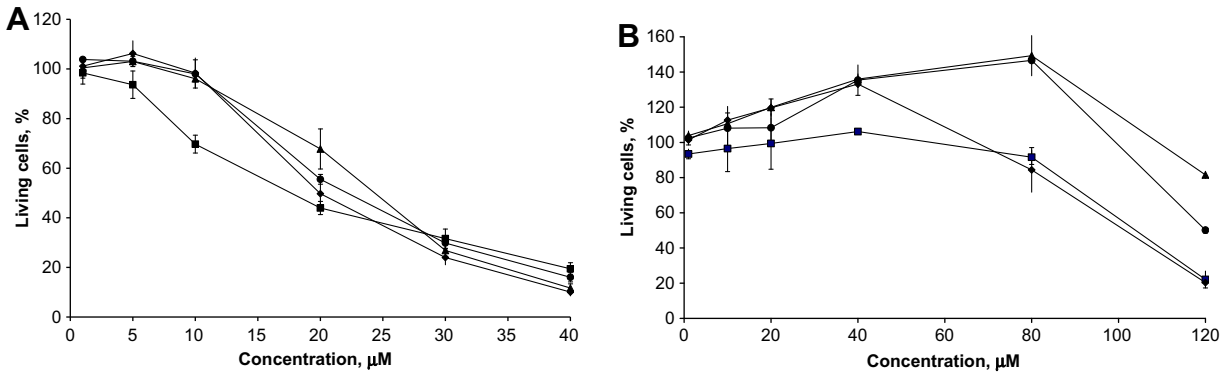


Figure 1. Viability of BHK IR-780 cells treated with cationic glycolipids: (A) cationic liposomes, (B) for 24 h. Cells were incubated with cationic glycolipids **7a–d** or with cationic liposome, consisting of glycolipids **7a–d** and DOPE (1:1) in the presence of 10% fetal bovine serum in the medium. Square for lipid **7a**, circle for lipid **7b**, up triangle for lipid **7c**, down triangle for lipid **7d**. Cell viability was calculated as the percentage of survival cells, the amount of living cells in controls was set as 100%, error bars represent standard deviations.

To study how the presence of DOPE can modify the cellular accumulation of FITC-ODN mediated by glycolipids **7a–d**, four types of lipoplexes were prepared using different ratios of FITC-ODN to cationic liposomes. Under serum-free conditions (Fig. 2A and B), the efficiency of FITC-ODN liposomal delivery was 3–30-fold higher than that of cationic glycolipids. The liposomes composed of lipid **7d** with *N*-methylpiperidinium 'head' and DOPE were the most efficient formulation over the entire used concentration range; the levels of FITC-ODN cellular delivery observed in the case of **7d**/DOPE liposomes (40%) were somewhat lower than those of Lipofectamine® 2000 (50%). Note that the cellular delivery of FITC-ODN mediated by cationic liposomes at a concentration of 20 μM only slightly depends on the concentration of ODN, thus, indicating that the ratio of 0.8 μM of FITC-ODN to 20 μM of liposomes (the N/P ratio of 1) provides an efficient cellular accumulation of this oligonucleotide. (Fig. 2A).

In the presence of serum, FITC-ODN cellular accumulation mediated by cationic liposomes (10 μM) was reduced two to threefolds, whereas the cellular accumulation of FITC-ODN at a liposome concentration of 20 μM was almost unaffected. Under these conditions (10% FBS and 20 μM liposome), the liposomes with glycolipid **7d** were the most active formulation providing the delivery of the oligonucleotide into 40% of the cells (Fig. 2C and D). Similarly to Lipofectamine, the cellular delivery of FITC-ODN by **7d**/DOPE liposomes

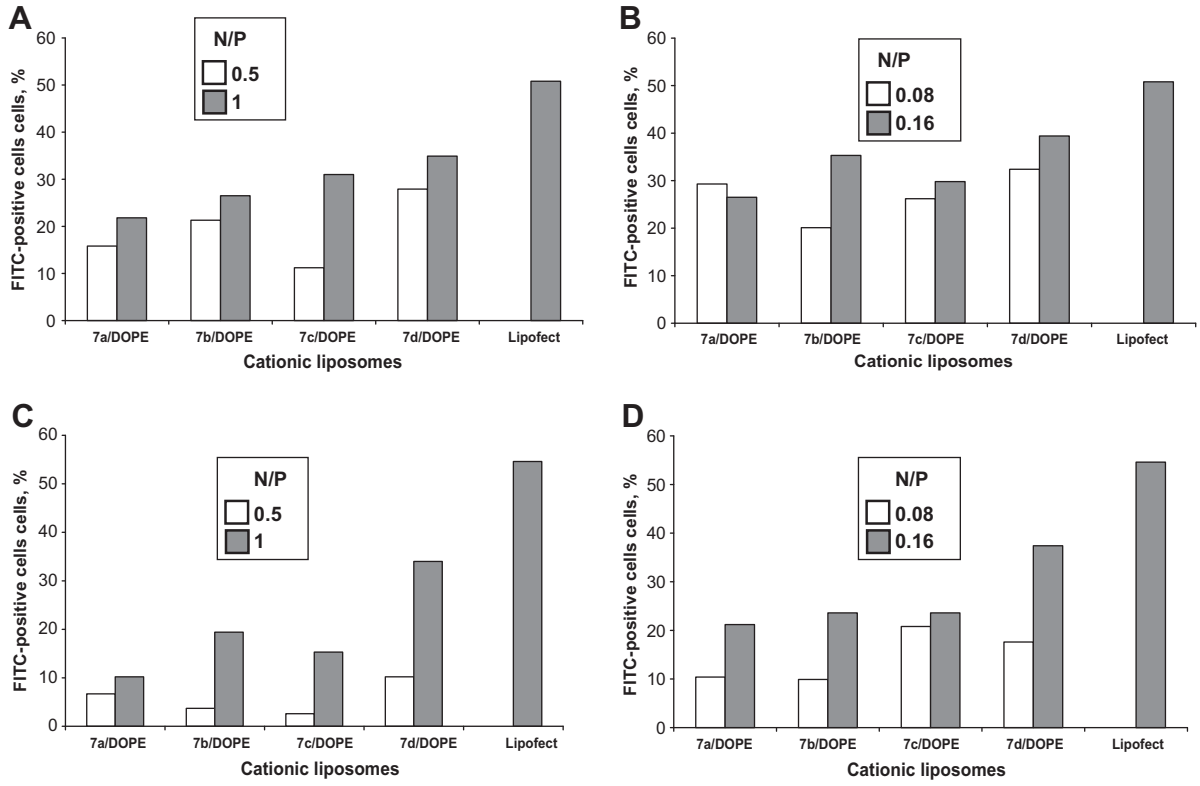


Figure 2. Cellular delivery of FITC-ODN into BHK cells in the absence (A and B) or presence (C and D) of 10% fetal bovine serum in the medium. Lipoplexes were composed of cationic liposomes (10 μM, white bar and 20 μM, gray bar) and FITC-ODN (A and C) ODN concentration of 0.8 μM; (B and D) liposome concentration of 5 μM) at various ratios. The percentage of FITC-positive cells was measured by FACS analysis after 4 h incubation with the corresponding FITC-ODN/liposome complex. Transfection with Lipofectamine® 2000 was carried out according to the manufacturer's protocol using 5 μM FITC-ODN. Standard deviations were within 5%.

(20 μM) remained constant both in the absence and in the presence of 10% FBS in the culture medium.

2.2.3. Delivery of siRNA into eukaryotic cells within siRNA/cationic liposome complexes

The ability of new cationic glycolipids and liposomes to mediate a cytosolic delivery of short nucleic acids was confirmed in the anti-EGFP-siRNA delivery experiments into the transgenic BHK IR780 cells expressing green fluorescent protein (EGFP). The chosen siRNA-EGFP (50 nM) is able to inhibit the EGFP expression in transgenic cells; thereby, a reduction in green fluorescence of BHK IR780 cells indicates the delivery activity of lipid or liposomal formulations. The percentage of EGFP-positive cells was estimated by flow cytometry at 72 h post-transfection. Untreated cells were used as a negative control (fluorescence intensity, 100%). The siRNA delivery with Lipofectamine® 2000 was used as a positive control. Cationic lipids insufficiently promoted the siRNA transfer into the cells. No gene-silencing effect was detected under serum-free conditions for complexes at lipids concentration 2.5 or 5 μM, and EGFP expression was reduced by 10–17% as compared with the control (100%) when the 10 μM of glycolipids was used. When the medium was supplemented with 10% FBS, the cellular delivery of siRNA by glycolipids **7a–d** was completely inhibited (primary data not shown).

In the case of the siRNA delivery with liposomal formulations, the reliable down-regulation of EGFP expression was observed (Table 1). Thus, in the absence of serum, cationic liposomes **7a**/DOPE, **7b**/DOPE, and **7c**/DOPE mediated the cellular delivery of siRNA, which resulted in the inhibition of EGFP synthesis by 27–30%. In the presence of serum, the efficiency was approximately halved for all tested liposomal formulations except for **7c**/DOPE. For liposomes **7c**/DOPE (40 μM), similar inhibition levels of EGFP expression were observed in the presence and in the absence of serum in the culture medium (29% and 31%, respectively). Under similar conditions, when Lipofectamine was used as a delivery agent, 77% inhibition of EGFP expression was observed.

In our study, we characterized the delivery of siRNA mediated by glycolipids/liposomal formulations by a down-regulation of EGFP expression appearing when siRNA reached the cytosol. Thus, the level of a biological effect depends on (1) cellular delivery of siRNA and (2) endosomal and/or liposomal release of siRNA. Our results showed that the cationic liposomes-containing lipids **7a–d** do deliver siRNA into the cells both in the presence and in the absence of serum. However, the down-regulation of EGFP expression in the case of Lipofectamine® 2000-mediated siRNA delivery was 2.5-fold higher. The results of our screening tests suggest that low levels of siRNA-mediated EGFP silencing can be associated with the endosomal or liposomal siRNA release rather than with the siRNA penetration across the cell membrane. Therefore, to enhance the biological effect of siRNA, it is necessary to perform deeper additional studies, including the optimization of liposomal compositions, siRNA-to-carrier ratio, and delivery conditions.

2.3. Physicochemical characteristics of liposomes and lipoplexes

Physicochemical properties of liposomes and their complexes with nucleic acids (lipoplexes) are important characteristics that can influence the transfection efficiency. We evaluated sizes and surface potentials of liposomal formulations alone and complexes formed with 25-mer FITC-ODN using the dynamic light scattering instrument equipped with ζ-sizing capacity.

The average size of the liposomes with cationic glycolipids **7b–d** was found to be identical ~100 nm. All liposomal formulations were positively charged, and ζ potentials were found to be within the range +13.07 to +34.38 mV (Table 2). The oligonucleotide added to the liposomes induced the formation of significantly bigger particles: for the liposomal formulations tested (**7a**/DOPE and **7d**/DOPE), formation of the particles of about 300 nm and 700 nm were observed. It is worse, noting that the initially opalescent solution of liposome/ODN complexes became turbid as the N/P ratio was increased from 0.08 to 1. The surface potential of the complexes prepared from liposomal formulation at N/P 1 was found to be negative. Biological experiments showed that the FITC-ODN cellular accumulation mediated by liposomal formulations was not affected by the size or the ζ potential of the lipoplexes. It seems that these two characteristic parameters of lipoplexes have midrange values and do not clarify the structure–activity relationships, therefore, to answer what supramolecular structures are formed by ODN and liposomal formulations, we have used the transmission electron microscopy (TEM).

Electron microscopic images (Fig. 3) showed the large heterogeneity in sizes and structures of liposomal formulations. The biggest amount was represented by homogeneous punctiforme particles with sizes from 20 to 100 nm (Fig. 3A). Liposomal formulations also contained closed lamellar structures within the range 50–120 nm (Fig. 3B) which can be sort to liposomes.[45] Interestingly, in the case of **7d**/DOPE, TEM picture showed the presence of a fingerprint pattern (Fig. 3C).[46]

Samples of liposomal formulations entrapping oligonucleotide at the N/P ratio of 1 (ODN (0.8 μM)–liposomes (20 μM)) contained globular aggregates with rod-like elements, and the average size was approximately 200–500 nm (Fig. 4A and B). The small lipid particles appeared to aggregate in the presence of the ODN to form multimodular complex particulates. Also we observed striped fine structures, which were in a close contact with a punctiforme

Table 1
siRNA mediated inhibition of EGFP expression in BHK IR-780 cells[a]

Delivery agent	Inhibition of EGFP expression[b] (%)							
Lipids concentration (μM)	5	10	20	40	5	10	20	40
10% FBS	–	–	–	–	+	+	+	+
7a/DOPE	12	9	12	ni[c]	ni	6	11	5
7b/DOPE	25	7	27	13	13	ni	9	9
7c/DOPE	21	3	16	29	ni	ni	ni	31
7d/DOPE	13	13	20	22	11	12	15	ni
Lipofectamine® 2000	76.9				85.6			

Flow cytometry data.

[a] BHK IR-780 cells were treated with lipoplex composed of siRNA (50 nM) and liposomal formulation (from 5 to 40 μM). Fluorescence of the cells was determined by FACS analysis after 72 h of incubation with the corresponding lipoplexes. Transfection with Lipofectamine® 2000 (10 μM) was carried out according to the manufacturer's protocol. Standard deviations were within 5%.

[b] Level of EGFP expression in the control cells (without treatment with siRNA/liposome) was set at 100%. Inhibition of EGFP expression was calculated as follows [(EGFP $\text{expression}_{\text{control}}$ − EGFP $\text{expression}_{\text{experiment}}$)/EGFP $\text{expression}_{\text{control}}$] × 100%.

[c] ni, no inhibition.

Table 2
Hydrodynamic diameters and ζ potentials of liposomal formulations and lipoplexes

Lipids	Liposomal formulations cationic lipids/DOPE (1:1 mol)		Liposomal formulation/ODN complexes[c]	
	Average size and polydispersity (nm)	ζ-Potential (mV)	Average size and polydispersity (nm)	ζ-potential (mV)
7a	72.9 ± 11.3[a] 261 ± 39.6[b]	+13.07	337.3 ± 27.9 742.7 ± 107.5	−9.82
7b	100.5 ± 18.7[a]	+23.12	nd	−13.27
7c	105.2 ± 20.8[a]	+30.73	nd	−18.91
7d	134.4 ± 24.1[a]	+34.38	361.0 ± 31.7 708.3 ± 136.9	−18.93

The surface potential of ODN/liposomal formulation at N/P ratio of 1 was found to be negative.
nd, not determined.
[a] Major population.
[b] Minor population.
[c] ODN (0.8 μM) was incubated with liposomal formulation (20 μM) (theoretical N/P ratio of 1) at 24 °C for 15 min.

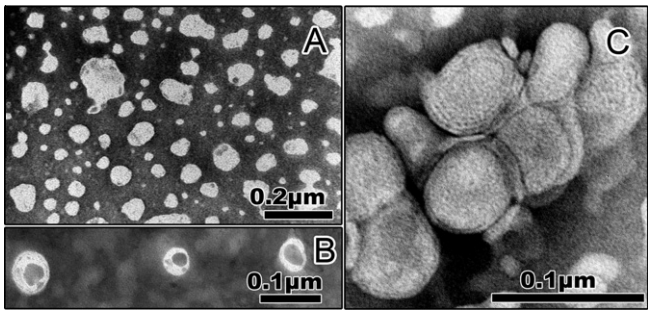


Figure 3. Morphology of the liposomal formulations. Figure shows TEM images (negative staining with phosphotungstic acid) of 1:1 **7a**/DOPE (A and C) and **7d**/DOPE (B) liposomal formulation. Lipid concentration is 20 μM. Notice, in C ordered regions, similar structures are also found in **7d**/DOPE liposome samples.

central region of lipoplexes (Fig. 4C). These stripes may be due to a fingerprint-like structure, similar to those of complexes formed by ODNs and cationic liposomes (cryo-EM data).[47,48] The fingerprint-striped structure is related to the formation of the lamellar lipid phase[47,49] but inverted hexagonal phases are also observed with lipoplexes resulting from the association of ODN[48] or DNA[46] with cationic liposomes. It is worse, noting that the fingerprint structure was predominantly found for **7d**/DOPE-ODN complexes. Decreasing the N/P ratio to 0.5 (ODN 0.8 μM–liposomes 10 μM), led to an increase in the sizes of the lipoplexes (Fig. 4D). At the same time, fingerprint-like fine structures were also clearly distinct (Fig. 4E). Lipoplexes **7d**/DOPE-ODN possessing higher transfection activity contained more fingerprint structures than lipoplexes formed by other liposomal formulations. This fact, that is, the presence of 'transfection promoting' fingerprint-like structures in **7d**/DOPE-ODN complexes, may explain the most pronounced transfection activity of liposomal formulation **7d**/DOPE as compared with other liposomal formulations tested.

Some distinct morphological differences were revealed for the lipoplexes prepared at low N/P ratios. TEM images of **7a**/DOPE-ODN lipoplexes at N/P of 0.16 showed the presence of clustered complexes formed by closely fine particles and the absence of striped structures (Fig. 4F). The complexes prepared from cationic liposomes and ODN at the minimal N/P ratio of 0.08 had shapeless structures bound with occasional spherical particles (Fig. 4G).

Thus, we synthesized new cholesteryl glucosides containing positively charged heterocyclic groups at the glucose C-6 atom. The goal of this study was to determine the most promising candidate for further in vitro and in vivo laboratory applications, among the tested compounds. Better understanding of the morphology of lipoplexes is important for further rational development of liposomal gene delivery systems. Our results give a starting point for the subsequent enhancement of the gene delivery activity of systems involving cationic cholesteryl glucosides.

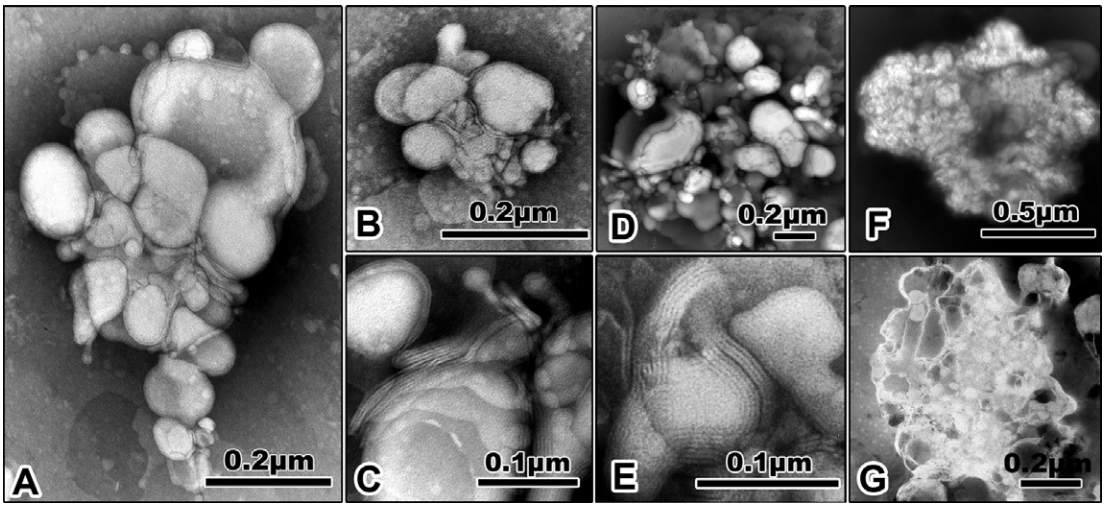


Figure 4. Morphology of the lipoplexes prepared from the ODN and liposomal formulations. (A and B) Lipoplexes composed of FITC-ODN and cationic liposomes **7a**/DOPE at the N/P ratios of 1 were of various sizes and shapes. (E) Striped fine structures, which were in close contact with a punctate central region of **7a**/DOPE-ODN complexes were observed. (C and F) Complexes composed of FITC-ODN and **7d**/DOPE at the N/P ratio of 0.5. Fingerprint-like fine structures were also clearly distinct. (D and F) Lipoplexes prepared at a low N/P (0.16 and 0.08) ratios showed clustered complexes and the absence of striped structures. TEM, negative staining with phosphotungstic acid.

3. Experimental

3.1. General methods

Toluene, ethyl methyl ketone, diethyl ether, and all amines were purified and dried by distillation from CaH_2 immediately before use. All solvents for column chromatography were distilled before use. Molecular sieves were activated at 180 °C under diminished pressure for 2 h. Thin layer chromatography was performed using pre-coated aluminum plates (Kieselgel 60 F_{254}, Merck), which were visualized with the phosphomolybdic acid–ceric sulfate reagent.[50] Flash column chromatography (FC) was performed on Kieselgel 60 (40–63 μm, Merck), silica gel (L 40–100 μm, Chemapol, Czech Republic), and Lichroprep® RP-18 (40–63 μm, Merck). Melting points were determined with a Boetius apparatus (Germany). Optical rotations were recorded on a Digytor Yasco DIP 360 (Japan) photoelectric spectropolarimeter as $[\alpha]_D$ values. ^1H and ^{13}C NMR spectra were recorded at 24 °C with Bruker MLS-200, Bruker AMX-400, and Bruker MSL-500 spectrometers in $CDCl_3$ as solvent unless otherwise stated. The signals of $SiMe_4$ ($\delta = 0.00$ ppm) and $CDCl_3$ ($\delta = 77.16$ ppm) were used as internal references. *J* values are given in Hertz. Signals were assigned by 2D proton–proton (COSY) and proton–carbon (HSQC) shift correlation spectra. Mass spectra were recorded with a VG ZAB-HSQ (VG Analytics) using 3-nitrobenzyl alcohol as a matrix (FAB MS), a Vision 2000 (Thermo BioAnalysis) time-of-flight mass spectrometer using 2,5-dihydroxybenzoic acid as a matrix (MALDI-TOF MS), or a MS 7 T APEX II (FT ICR, Bruker-Daltonics) high-resolution mass spectrometer in the positive mode (HRESIMS).

3.2. Cholest-5-en-3β-yl 2,3,4,6-tetra-*O*-acetyl-D-glucopyranoside (2)

3.2.1. Procedure using mercury cyanide

$HgCN_2$ (1.5 g, 6.09 mmol) and 4 Å molecular sieves (2.0 g) were added to a soln of cholesterol (4.7 g, 12.19 mmol) in dry toluene (26 mL) at 20 °C under stirring. After 30 min, the soln of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**1**) (2.5 g, 6.09 mmol) in dry toluene (8 mL) was added dropwise within 30 min. After 7 h at 100 °C, the reaction mixture was cooled to ambient temperature, filtered, and washed with 20% aq KI (2 × 5 mL) and water (2 × 5 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under diminished pressure. The residue was purified by FC (40/100 μm, 4:1 petroleum ether–diethyl ether,) to give α-(**2a** 0.84 g, 19%) and β-anomers (**2b** 1.92 g, 44%).

3.2.2. Procedure using cadmium carbonate

A mixture of cholesterol (7.37 g, 19.06 mmol) and $CdCO_3$ (1.64 g, 9.53 mmol) in dry toluene (80 mL) was refluxed in a Soxhlet extractor filled with activated granular silica gel for 3 h. Bromide **1** (3.51 g, 8.54 mmol) was then added portionwise within 1 h, and the mixture was additionally refluxed for 3 h and cooled. Cadmium salts were filtered off, the solvent was evaporated, and the residue was purified on silica gel to give α-(**2a** 0.06 g, 1%) and β-anomers (**2b** 3.15 g, 51%) as white solids.

α-Anomer (**2a**): mp 188–190 °C, lit.[51] 193–195 °C; $[\alpha]_D^{25}$ +88.05 (*c* 1.0, $CHCl_3$), lit.[51] $[\alpha]_D^{20}$ +92, lit.[52] $[\alpha]_D^{20}$ +88; ^1H NMR (200 MHz): δ 5.42 (dd, 1H, $J_{3,2}$ 9.4 Hz, $J_{3,4}$ 10.2 Hz, H-3 Glc), 5.24–5.32 (m, 1H, H-6), 5.16 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1 Glc), 4.96 (t, 1H, $J_{4,3}$, $J_{4,5}$ 9.4 Hz, H-4 Glc), 4.74 (dd, 1H, $J_{2,1}$ 3.8 Hz, $J_{2,3}$10.2 Hz, H-2 Glc), 3.95–4.21 (m, 3H, H-5 Glc, $H_{a,b}$-6 Glc), 3.28–3.41 (m, 1H, H-3), 2.17–2.30 (m, 2H, $H_{a,b}$-4), 2.02 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H) and 1.95 (s, 3H, 4 $COCH_3$), 0.97–1.92 (m, 26H, H cholesterol), 0.95 (s, 3H, CH_3-19), 0.84 (d, 3H, $J_{21,20}$ 6.4 Hz, CH_3-21), 0.79 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.4 Hz, CH_3-26,27), 0.62 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz): δ 170.56, 170.25, 169.40, and 169.23, (4 *C*(O)CH_3), 140.56 (C-5), 122.15 (C-6), 94.47 (C-1 Glc), 80.14 (C-3), 71.31 (C-3 Glc), 70.45 (C-2 Glc), 69.15 (C-4 Glc), 67.53 (C-5 Glc), 62.38 (C-6 Glc), 56.96 (C-14), 56.43 (C-17), 50.44 (C-9), 42.50 (C-13), 39.97 (C-4), 39.67 (C-12), 39.08 (C-24), 37.17 (C-1), 36.88 (C-10), 36.37 (C-22), 35.85 (C-20), 32.06 (C-7,8), 29.64 (C-2), 28.28 (C-16), 28.10 (C-25), 24.38 (C-15), 23.98 (C-23), 22.84 and 22.66 (C-26,27), 21.30 (C-11), 20.64, 20.62, 20.56 (4 C(O)*C*H_3), 19.42 (C-19), 18.87 (C-21), 11.98 (C-18); $C_{41}H_{64}O_{10}$ (716.95, 716.4499), MALDI-TOFMS: *m/z* 739.9 $[M+Na]^+$; Anal. Calcd for $C_{41}H_{64}O_{10}$: C, 68.69; H, 9.00. Found: C, 68.71; H, 8.58.

β-Anomer (**2b**): mp 152–154 °C, lit.[51] 157–159 °C; $[\alpha]_D^{25}$ −20.15 (*c* 1.0, $CHCl_3$), lit.[51] $[\alpha]_D^{20}$ −26; ^1H NMR (200 MHz): δ 5.26–5.31 (m, 1H, H-6), 5.14 (t, 1H, $J_{3,2}$, $J_{3,4}$ 9.4 Hz, H-3 Glc), 4.98 (t, 1H, $J_{4,3}$, $J_{4,5}$ 9.4 Hz, H-4 Glc), 4.89 (dd, 1H, $J_{2,1}$ 7.7 Hz, $J_{2,3}$ 9.4 Hz, H-2 Glc), 4.52 (d, 1H, $J_{1,2}$ 7.7 Hz, H-1 Glc), 4.19 (dd, 1H, $J_{6b,5}$ 2.5 Hz, $J_{6b,6a}$ 12.0 Hz, H_b-6 Glc), 4.03 (dd, 1H, $J_{6a,5}$ 5.0 Hz, $J_{6a,6b}$ 12.0 Hz, H_a-6 Glc), 3.61 (ddd, 1H, $J_{5,6b}$ 2.5 Hz, $J_{5,6a}$ 5.0 Hz, $J_{5,4}$ 9.4 Hz, H-5 Glc), 3.32–3.49 (m, 1H, H-3), 2.04–2.19 (m, 2H, $H_{a,b}$-4), 2.01 (s, 3H), 1.98 (s, 3H), 1.95 (s, 3H), and 1.93 (s, 3H, 4 $COCH_3$), 1.02–1.92 (m, 26H, H cholesterol), 0.92 (s, 3H, CH_3-19), 0.84 (d, 3H, $J_{21,20}$ 6.4 Hz, CH_3-21), 0.80 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.4 Hz, CH_3-26,27), 0.61 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz): δ 170.55, 170.26, 169.41, and 169.23 (4 *C*(O)CH_3), 140.67 (C-5), 122.22 (C-6), 99.88 (C-1 Glc), 80.18 (C-3), 73.30 (C-3 Glc), 71.98 (C-2,4 Glc), 69.11 (C-5 Glc), 62.46 (C-6 Glc), 57.06 (C-14), 56.54 (C-17), 50.55 (C-9), 42.61 (C-13), 40.04 (C-12), 39.75 (C-24), 39.16 (C-4), 37.45 (C-1), 36.95 (C-10), 36.44 (C-22), 35.93 (C-20), 32.17 (C-7,8), 29.71 (C-2), 28.35 (C-16), 28.13 (C-25), 24.46 (C-15), 24.05 (C-23), 22.88 and 22.66 (C-26,27), 21.30 (C-11), 20.72, 20.70, 20.64 (4 C(O)*C*H_3), 19.50 (C-19), 18.94 (C-21), 12.03 (C-18); $C_{41}H_{64}O_{10}$ (716.95, 716.4499), MALDI-TOFMS: *m/z* 739.6 $[M+Na]^+$; Anal. Calcd for $C_{41}H_{64}O_{10}$: C, 68.69; H, 9.00. Found: C, 68.86; H, 8.67.

3.3. Cholest-5-en-3-yl β-D-glucopyranoside (3)

NaOMe (0.2 N, 8 mL) solution was added to the soln of **2b** (1.92 g, 2.68 mmol) in MeOH (20 mL) and $CHCl_3$ (8 mL). After 3 h at 20 °C. the reaction mixture was treated with Dowex 50w × 8 (H^+), filtered, and concentrated under diminished pressure. The crude product was recrystallized from MeOH to give the desired product **3** as a white solid (1.47 g, 90%); mp 265–267 °C (from MeOH); $[\alpha]_D^{25}$ −41.42 (*c* 0.8, $CHCl_3$), lit.[53] $[\alpha]_D^{20}$ −48.5 (*c* 2.45, pyridine); ^1H NMR (400 MHz, Py-d5): δ 5.32–5.37 (m, 1H, H-6), 5.05 (d, 1H, $J_{1,2}$ 7.6 Hz, H-1 Glc), 4.55 (dd, 1H, $J_{6b,5}$ 2.5 Hz, $J_{6b,6a}$ 11.9 Hz, H_b-6 Glc), 4.40 (dd, 1H, $J_{6a,5}$ 5.3 Hz, $J_{6a,6b}$ 11.9 Hz, H_a-6 Glc), 4.22–4.32 (m, 2H, H-3 Glc, H-5 Glc), 4.04 (dd, 1H, $J_{2,1}$ 7.6, $J_{2,3}$ 8.6 Hz, H-2 Glc), 3.88–4.00 (m, 2H, H-3, H-4 Glc), 2.64–2.80 (m, 1H, H_b-4), 2.38–2.56 (m, 1H, H_a-4), 0.99–2.20 (m, 24H, H cholesterol), 0.96 (d, 3H, $J_{21,20}$ 6.4 Hz, CH_3-21), 0.93 (s, 3H, CH_3-19), 0.89 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6 Hz, CH_3-26,27), 0.65 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz): δ 140.99, 121.98, 102.65, 78.63, 78.52, 78.19, 75.37, 71.75, 62.89, 56.90, 56.44, 50.44, 42.55, 40.04, 39.79, 39.41, 37.56, 36.99, 36.10, 36.55, 32.25, 32.13, 30.33, 28.57, 28.30, 24.56, 24.21, 23.00, 22.75, 21.36, 19.49, 19.01, 12.06; $C_{33}H_{56}O_6$ (548.00, 548.4077), MALDI-TOFMS: *m/z* 571.7 $[M+Na]^+$; Anal. Calcd for $C_{33}H_{56}O_6$: C, 72.22; H, 10.29. Found: C, 72.11; H, 10.19.

3.4. Cholest-5-en-3β-yl 2,3,4-tri-*O*-acetyl-6-*O*-methanesulfonyl-β-D-glucopyranoside (4)

Methanesulfonyl chloride (0.06 g, 0.55 mmol) in anhyd pyridine (1.8 mL) cooled to −15 °C was added rapidly to the cooled soln of **3** (0.3 g, 0.55 mmol) in anhyd pyridine (25 mL). After 20 h at −15 °C, the reaction mixture was warmed to 20 °C, Ac_2O (0.31 mL, 3.3 mmol) was added, and the reaction mixture was kept for 3 h.

The organic solvents were removed under diminished pressure, and the resulting residue was purified by FC (40/100 μ, 7:3 petroleum ether–Et_2O) to give **4** as a white crystalline solid (0.29 g, 70%); mp 170–172 °C; $[\alpha]_D^{25}$ −15.35 (*c* 1.0, $CHCl_3$); ^1H NMR (200 MHz): δ 5.31–5.36 (m, 1H, H-6), 5.19 (t, 1 H, $J_{3,2}$, $J_{3,4}$ 9.4 Hz, H-3 Glc), 5.01 (dd, 1H, $J_{4,3}$ 9.4 Hz, $J_{4,5}$ 9.8 Hz, H-4 Glc), 4.92 (dd, 1H, $J_{2,1}$ 8.1 Hz, $J_{2,3}$ 9.4 Hz, H-2 Glc), 4.59 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1 Glc), 4.27 (d, 2H, $J_{6a,5}$, $J_{6b,5}$ 3.8 Hz, $H_{a,b}$-6 Glc), 3.75 (dt, 1 H, $J_{5,6a}$, $J_{5,6a}$ 3.8 Hz, $J_{5,4}$ 9.8 Hz, H-5 Glc), 3.36–3.52 (m, 1H, H-3), 3.03 (s, 3H, SCH_3), 2.07–2.21 (m, 2H, $H_{a,b}$-4), 2.03 (s, 6H) and 1.98 (s, 3H, 3 $COCH_3$), 0.99–1.97 (m, 26H, H cholesterol), 0.97 (s, 3H, CH_3-19), 0.88 (d, 3H, $J_{20,21}$ 6.4 Hz, CH_3-21), 0.84 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.4 Hz, CH_3-26,27), 0.66 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz): δ 170.12, 169.49 and 169.12 (3*C*(O)CH_3), 140.34 (C-5), 122.33 (C-6), 99.80 (C-1 Glc), 80.25 (C-3), 72.90 (C-3 Glc), 71.80 (C-2 Glc), 71.69 (C-4 Glc), 68.78 (C-5 Glc), 67.31 (C-6 Glc), 56.91 (C-14), 56.43 (C-17), 50.41 (C-9), 42.50 (C-13), 39.93 (C-12), 39.67 (C-24), 39.05 (C-4), 37.80 (SCH_3), 37.36 (C-1), 36.84 (C-10), 36.37 (C-22), 35.85 (C-20), 32.07 (C-7, 8), 29.75 (C-2), 28.28 (C-16), 28.06 (C-25), 24.38 (C-15), 23.98 (C-23), 22.84 and 22.62 (C-26, 27), 21.22 (C-11), 20.56 (3 C(O)*C*H_3), 19.42 (C-19), 18.87 (C-21), 11.96 (C-18); $C_{40}H_{64}O_{11}S$ (753.00, 752.4169), MALDI-TOFMS: *m/z* 776.0 $[M+Na]^+$, 792.3 $[M+K]^+$; Anal. Calcd for $C_{40}H_{64}O_{11}S$: C, 63.80; H, 8.57; S, 4.26. Found: C, 63.63; H, 8.37; S, 4.16.

3.5. Cholest-5-en-3β-yl 2,3,4-tri-*O*-acetyl-6-deoxy-6-pyridinio-β-D-glucopyranoside methanesulfonate (5a)

The soln of **4** (0.08 g, 0.11 mmol) in anhyd pyridine (2 mL) was heated for 9 h at 115 °C. The residue obtained after removing the solvent and traces of pyridine (0.1 Torr, 40 °C) was recrystallized from dry Et_2O to give **5a** as a slightly beige solid (0.07 g, 78%); mp 247–249 °C, $[\alpha]_D^{25}$ −12.69 (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz): δ 9.20 (m, 2H), 8.48 (m, 1H) and 8.03 (m, 2H, $C_5H_5N^+$), 5.30–5.44 (m, H, H-6), 5.26–5.30 (m, 2H, H-2 Glc, H-3 Glc), 4.76–4.87 (m, 3H, H-4 Glc, $H_{a,b}$-6 Glc), 4.60 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1 Glc), 4.23–4.26 (m, 1H, H-5 Glc), 3.17–3.25 (m, 1H, H-3), 2.69 (s, 3H, SCH_3), 2.21 (s, 3H, $COCH_3$), 2.17–2.20 (m, 1H, H-4_b), 2.07–2.12 (m, 1H, H-4_a), 2.02 (s, 3H) and 1.99 (s, 3H, 2 $COCH_3$), 1.92–1.95 (m, 3H, H_b-7, $H_{a,b}$-12), 1.72–1.86 (m, 3H, H_b-1, H_b-2, H_b-16), 0.95–1.59 (m, 20H, H cholesterol), 0.94 (s, 3H, H-19), 0.91 (d, 3H, $J_{21,20}$ 6.5 Hz, CH_3-21), 0.86 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6 Hz, CH_3-26,27), 0.67 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz): δ 170.85, 169.82 and 169.38 (*C*(O)CH_3), 139.83 (C-5), 146.95, 145.70 and 127.85 ($C_5H_5N^+$),122.66 (C-6), 99.88 (C-1 Glc), 80.58 (C-3), 72.50 (C-3 Glc), 71.72 (C-2 Glc), 71.47 (C-4 Glc), 69.67 (C-5 Glc), 61.54 (C-6 Glc), 56.88 (C-14), 56.40 (C-17), 50.26 (C-9), 42.51 (C-13), 39.90 (C-12), 39.79 (SCH_3), 39.67 (C-24), 38.94 (C-4), 37.21 (C-1), 36.77 (C-10), 36.37 (C-22), 35.89 (C-20), 32.03 (C-7, 8), 29.71 (C-2), 28.32 (C-16), 28.13 (C.25), 24.42 (C-15), 24.02 (C-23), 22.88 and 22.66 (C-26, 27), 21.22 (C-11), 21.04, 20.75 and 20.67 (3 C(O)*C*H_3), 19.24 (C-19), 18.87 (C-21), 12.00 (C-18); $C_{45}H_{69}NO_{11}S$ (832.10, 831.4591), MALDI-TOFMS: *m/z* 737.5 $[M-MsO+H]^+$; HRESIMS: found 736.4775, calcd for $[M-MsO]^+$ 736.4793.

3.6. Cholest-5-en-3β-yl 2,3,4-tri-*O*-acetyl-6-deoxy-6-(*N*-methylimidazolio)-β-D-glucopyranoside methanesulfonate (5b)

A mixture of **4** (0.075 g, 0.10 mmol) and *N*-methylimidazole (0.4 mL, 5 mmol) in dry ethyl methyl ketone (2 mL) was kept for 19 h at 100 °C. The reaction soln was concentrated under diminished pressure. The crude product was purified by FC (40/100 μ, 7:3 $CHCl_3$–MeOH) to give **5b** as an amorphous solid (0.066 g, 80%); $[\alpha]_D^{25}$ −30.03 (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz): δ 10.47 (s, 1H), 7.44 (s, 1H) and 7.19 (s, 1H, H imidazole), 5.33–5.37 (m, 1 H, H-6), 5.24 (t, 1 H, $J_{3,2}$, $J_{3,4}$ 9.6 Hz, H-3 Glc), 4.85 (t, 1H, $J_{4,3}$, $J_{4,5}$ 9.6 Hz, H-4 Glc), 4.68–4.79 (m, 4H, H-1 Glc, H-2 Glc, $H_{a,b}$-6 Glc), 4.06–4.20 (m, 1H, H-5 Glc), 4.03 (s, 3H, CH_3N^+), 3.40–3.47 (m, 1H, H-3), 2.73 (s, 3H, SCH_3), 2.26 (s, 3H, $COCH_3$), 2.17–2.21 (m, 1H, H_b-4), 2.03 (s, 3H, $COCH_3$), 1.99 (m, 1H, H_a-4), 1.97 (s, 3H, $COCH_3$), 0.95–1.95 (m overlapped with s at 0.98, 26H, H cholesterol), 0.98 (s, 3H, CH_3-19), 0.91 (d, 3H, $J_{21,20}$ 6.5 Hz, CH_3-21), 0.86 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6 Hz, CH_3-26,27), 0.67 (s, 3H, CH_3-18); ^{13}C NMR (75 MHz, APT): δ 170.97 (+), 169.95 (+) and 169.56 (+, 3*C*(O)CH_3), 139.82 (+, C-5), 122.78 (−, C-6), 123.67 (−) and 122.31 (−, CH=CHN=CH), 99.83 (−, C-1 Glc), 80.63 (−, C-3), 72.29 (−, C-3 Glc), 71.66 (−, C-2 Glc), 71.29 (−, C-4 Glc), 68.31 (−, C-5 Glc), 56.84 (−, C-14), 56.28 (−, C-17), 50.20 (−, C-9 and CH_3N^+),49.61 (+, C-6 Glc), 42.41 (+, C-13), 39.82 (+, C-12), 39.63 (+, C-24), 39.00 (+, C-4), 37.24 (+, C-1), 36.82 (−, SCH_3), 36.75 (+, C-10), 36.30 (+, C-22), 35.90 (−, C-20), 32.02 (+, C-7), 31.95 (−, C-8), 29.80 (+, C-2), 28.35 (+, C-16), 28.14 (−, C-25), 24.40 (+, C-15), 23.95 (+, C-23), 23.00 and 22.70 (−, C-26, 27), 21.47 (−, C(O)*C*H_3), 21.19 (+, C-11), 20.82 (−) and 20.66 (−, 2 C(O)*C*H_3), 19.48 (−, C-19), 18.85 (−, C-21), 12.00 (−, C-18); $C_{44}H_{70}N_2O_{11}S$ (835.10, 834.4700), MALDI-TOFMS: *m/z* 739.9 $[M-MsO+H]^+$; HRESIMS: found 739.4876, calcd for $[M-MsO]^+$ 739.4892.

3.7. Cholest-5-en-3β-yl 2,3,4-tri-O-acetyl-6-deoxy-6-(*N*-methylmorpholinio)-β-D-glucopyranoside iodide (5c)

3.7.1. Cholest-5-en-3 β-yl 2,3,4-tri-*O*-acetyl-6-deoxy-6-morpholino-β-D-glucopyranoside (6a)

A mixture of **4** (0.08 g, 0.102 mmol) and anhyd morpholine (2 mL) was heated for 9 h at 70 °C. The reaction mixture was cooled to 20 °C to add Ac_2O (0.05 mL, 0.53 mmol). After 3 h, the mixture was concentrated under diminished pressure and purified by FC (40/63 μ, $CHCl_3$) to yield **6a** as a white solid (0.048 g, 52%); mp 211–213 °C; $[\alpha]_D^{25}$ −17.70 (*c* 1.0, $CHCl_3$); ^1H NMR (200 MHz): δ 5.28–5.34 (m, 1H, H-6), 5.19 (t, 1H, $J_{3,2}$, $J_{3,4}$ 9.4 Hz, H-3 Glc), 5.01 (dd, 1H, $J_{4,3}$ 9.4 Hz, $J_{4,5}$ 9.8 Hz, H-4 Glc), 4.92 (dd, 1H, $J_{2,1}$ 8.1 Hz, $J_{2,3}$ 9.4 Hz, H-2 Glc), 4.51 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1 Glc), 3.54–3.69 (m, 5H, H-5 Glc, CH_2OCH_2), 3.32–3.49 (m, 1H, H-3), 2.43–2.52 (m, 6H, $H_{a,b}$-6 Glc, CH_2NCH_2), 2.05–2.25 (m, 2H, $H_{a,b}$-4), 2.00 (s, 3H), and 1.99 (s, 3H) and 1.96 (s, 3H, 3 $COCH_3$), 0.92–1.94 (m overlapped with s at 0.92 and 0.98, 26H, H cholesterol), 0.98 (s, 3H, CH_3-19), 0.95 (d, 3H, $J_{21,20}$ 6.4 Hz, CH_3-21), 0.83 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.4 Hz, CH_3-26,27), 0.64 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz): δ 170.30, 169.52 and 169.30 (3 *C*(O)CH_3), 140.64 (C-5), 122.22 (C-6), 99.80 (C-1 Glc), 80.10 (C-3), 73.45 (C-3 Glc), 71.75 (C-2 Glc), 72.05 (C-4 Glc), 70.99 (C-5 Glc), 67.05 (CH_2NCH_2), 59.37 (C-6 Glc), 57.02 (C-14), 56.51 (C-17), 54.71 (CH_2OCH_2), 50.52 (C-9), 42.62 (C-13), 40.04 (C-12), 39.74 (C-24), 39.17 (C-4), 37.54 (C-1), 36.96 (C-10), 36.44 (C-22), 35.93 (C-20), 32.17 (C-7, 8), 29.82 (C-2), 28.29 (C-16), 28.17 (C-25), 24.46 (C-15), 24.05 (C-23), 22.91 and 21.69 (C-26, 27), 21.30 (C-11), 20.87, 20.75 and 20.67 (3 C(O)*C*H_3), 19.49 (C-19), 18.95 (C-21), 12.04 (C-18); $C_{43}H_{69}NO_9$ (744.01, 743.4972), FABMS: *m/z* 744.5 $[M+H]^+$; Anal. Calcd for $C_{43}H_{69}NO_9 \cdot H_2O$: C, 67.78; H, 9.39; N, 1.84. Found: C, 67.77; H, 9.49; N, 1.40.

3.7.2. Quaternarization of amine 6a

A mixture of **6a** (0.050 g, 0.067 mmol) and freshly distilled MeI (2 mL) was kept for 3 h at 40 °C. The excess of MeI was removed under diminished pressure, and the residue was recrystallized from dry Et_2O to give **5c** as a yellow amorphous solid (0.049 g, 82%); $[\alpha]_D^{25}$ −13.72 (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz): δ 5.48 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1 Glc), 5.41–5.45 (m, 1H, H-6), 5.34 (t, 1H, $J_{3,2}$, $J_{3,4}$ 9.6 Hz, H-3 Glc), 4.98–5.04 (m, 1H, H-5 Glc), 4.96 (dd, 1H, $J_{2,1}$ 8.1 Hz, $J_{2,3}$ 9.6 Hz, H-2 Glc), 4.90 (t, 1H, $J_{4,3}$, $J_{4,5}$ 9.6 Hz, H-4 Glc), 3.95–4.17 (m, 7H, CH_2OCH_2, CH_2N^+, H_b-6 Glc), 3.87 (s, 3H, CH_3N^+), 3.76–3.85 (m, 2H, CH_2N^+), 3.68 (dd, 1H, $J_{6a,5}$ 9.4 Hz,

$J_{6a,6b}$13.9 Hz, H_a-6 Glc), 3.51–3.58 (m, 1H, H-3), 2.43–2.48 (m, 1H, H_b-4), 2.30 (s, 3H, $COCH_3$), 2.10–2.18 (m, 1H, H_a-4), 2.07 (s, 3H) and 2.00 (s, 3H, 2 $COCH_3$), 1.92–1.99 (m, 3H, H_b-7, $H_{a,b}$-12), 1.04–1.84 (m, 23H, H cholesterol), 1.00 (s, 3H, CH_3-19), 0.97 (m, 1H, H-9), 0.95 (d, 3H, $J_{21,20}$ 6.5 Hz, CH_3-21), 0.87 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6 Hz, CH_3-26, 27), 0.67 (s, 3H, CH_3-18); ^{13}C NMR (125 MHz, APT): δ 170.66 (+), 170.17 (+) and 169.54 (+, 3 $C(O)CH_3$), 139.63 (+, C-5), 123.14 (–, C-6), 98.67 (–, Glc-1), 80.29 (–, C-3), 72.10 (–, C-3Glc), 71.07 (–, C-2 Glc), 69.99 (–, C-4 Glc), 67.70 (–, C-5 Glc), 64.20 (+, C-6 Glc), 62.70 (+) and 61.59 (+, $CH_2N^+CH_2$), 60.84 (+, CH_2OCH_2), 56.88 (–, C-14), 56.25 (–, C-17), 50.11 (–, N^+CH_3), 49.98 (–, C-9), 42.51 (+, C-13), 39.92 (+, C-12), 39.72 (+, C-24), 39.01 (+, C-4), 37.24 (+, C-1), 36.79 (+, C-10), 36.39 (+, C-22), 35.97 (–, C-20), 32.13 (+, C-7), 32.07 (–, C-8), 29.77 (+, C-2), 28.50 (+, C-16), 28.20 (–, C-25), 24.47 (+, C-15), 24.03 (+, C-23), 22.99 and 21.74 (–, C-26, 27), 22.56 (–, C(O)CH_3), 21.25 (+, C-11), 20.87 (–) and 20.78 (–, 2 C(O)CH_3), 19.51 (–, C-19), 18.91 (–, C-21), 12.04 (–, C-18); $C_{44}H_{72}INO_9$ (885.96, 885.4252), HRESIMS: found 758.5197, calcd for $[M-I]^+$ 758.5201.

3.8. Cholest-5-en-3β-yl 2,3,4-tri-*O*-acetyl-6-deoxy-6-(*N*-methylpiperidinio)-β-D-glucopyranoside iodide (5d)

3.8.1. Cholest-5-en-3β-yl 2,3,4-tri-*O*-acetyl-6-deoxy-6-piperidino-β-D-glucopyranoside (6b)

Methanesulfonate **4** (0.07 g, 0.093 mmol) was treated with piperidine (1 mL) at 60 °C for 3 h and Ac_2O (0.05 mL, 0.53 mmol) as described for **6a**. The FC on silica gel (40/63 μm, $CHCl_3$) gave **6b** (0.04 g, 58%) as a white solid: mp 198–200 °C, $[\alpha]_D^{25}$ −24.25 (*c* 1.0, $CHCl_3$); ^{1}H NMR (200 MHz): δ 5.27–5.34 (m, 1H, H-6), 5.12 (t, 1H, $J_{3,2}$, $J_{3,4}$ 9.4 Hz, H-3 Glc), 5.01 (dd, 1H, $J_{4,3}$ 9.4 Hz, $J_{4,5}$ 9.8 Hz, H-4 Glc), 4.86 (dd, 1H, $J_{2,1}$ 8.1 Hz, $J_{2,3}$ 9.4 Hz, H-2 Glc), 4.49 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1 Glc), 3.58 (dt, 1H, $J_{5,6}$ 4.7 Hz, $J_{5,4}$ 9.8 Hz, H-5 Glc), 3.31–3.48 (m, 1H, H-3), 2.43 (d, 2H, $J_{6,5}$ 4.7 Hz, H-6 Glc), 2.26–2.40 (m, 4H, CH_2NCH_2), 2.02–2.21 (m, 2H, $H_{a,b}$-4), 2.00 (s, 3H), 1.98 (s, 3H) and 1.96 (s, 3H, 3 $COCH_3$), 1.00–1.94 (m, 32H, H cholesterol, $(CH_2)_3$), 0.93 (s, 3H, CH_3-19), 0.86 (d, 3H, $J_{21,20}$ 6.4 Hz, CH_3-21), 0.81 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.8 Hz, CH_3-26,27), 0.62 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz): δ 169.34, 169.63 and 170.67 (3 $C(O)CH_3$), 140.74 (C-5), 122.14 (C-6), 99.73 (C-1 Glc), 80.07 (C-3), 73.52 (C-3 Glc), 72.94 (C-2 Glc), 72.13 (C-4 Glc), 71.28 (C-5 Glc), 60.03 (C-6 Glc), 57.06 (C-14), 56.51 (C-17), 55.59 (CH_2NCH_2), 50.52 (C-9), 42.61 (C-13), 40.04 (C-12), 39.75 (C-24), 39.23 (C-4), 37.54 (C-1), 36.96 (C-10), 36.44 (C-22), 35.93 (C-20), 32.17 (C-7, 8), 29.79 (C-2), 28.39 (C-16), 28.17 (C-25), 26.26 ($CH_2CH_2CH_2$), 24.50 (C-15), 24.34 ($CH_2CH_2CH_2$), 24.05 (C-23), 22.91 and 22.69 (C-26, 27), 21.30 (C-11), 20.89, 20.78, 20.71 (3 C(O)CH_3), 19.50 (C-19), 18.95 (C-21), 12.04 (C-18); $C_{44}H_{71}NO_8$ (742.04, 741.5180), FABMS: *m/z* 742.5 $[M+H]^+$; Anal. Calcd for $C_{44}H_{71}NO_8$: C, 71.22; H, 9.64; N, 1.89. Found: C, 71.37; H, 9.80; N, 1.81.

3.8.2. Quaternization of amine 6b

Cholest-5-en-3β-yl 2,3,4-tri-*O*-acetyl-6-deoxy-6-(*N*-methylpiperidinio)-β-D-glucopyranoside iodide **(5d)** was prepared by the procedure described for compound **5c** using **6b** (0.030 g, 0.04 mmol) and MeI (0.6 mL). The product was purified by the FC on silica gel (40/63 μ, 10:1 $CHCl_3$–MeOH) to yield **5d** as a yellow solid (0.022 g, 72%): mp 142–144 °C $[\alpha]_D^{25}$ −15.67 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz) δ 5.42–5.45 (m, 1H, H-6), 5.40 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1 Glc), 5.31 (br t, 1H, $J_{3,2}$, $J_{3,4}$ 9.3 Hz, H-3 Glc), 4.95 (dd, 1H, $J_{2,1}$ 8.1 Hz, $J_{2,3}$ 9.7 Hz, H-2 Glc), 4.82–4.89 (m, 2H, H-4 Glc, H-5 Glc), 3.96–4.09 (m, 2H, CH_2N^+), 3.73–3.88 (m, 2H, CH_2N^+), 3.68–3.70 (m, 1H, H_b-6 Glc), 3.66 (s, 3H, CH_3N^+), 3.51–3.58 (m, 2H, H-3, H_a-6 Glc), 2.38–2.43(m, 1H, H_b-4), 2.28 (s, 3H, $COCH_3$), 2.09–2.17 (m, 1H, H_a-4), 2.06 (s, 3H) and 1.99 (s, 3H, 2 $COCH_3$), 1.79–2.02 (m overlapped with s at 1.99, 9H, cholesterol, $CH_2CH_2CH_2$), 0.99–1.84 (m, 25H, H cholesterol, $CH_2CH_2CH_2$), 0.97 (s, 3H, CH_3-19), 0.95 (m, 1H, H-9), 0.92 (d, 3H, $J_{21,20}$ 6.5 Hz, CH_3-21), 0.86 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6 Hz, CH_3-26, 27), 0.67 (s, 3H, CH_3-18); ^{13}C NMR (125 MHz, APT): δ 169.57 (+), 170.04 (+) and 170.56 (+, 3 $C(O)CH_3$), 139.64 (+, C-5), 123.04 (–, C-6), 98.64 (–, C-1 Glc), 80.23 (–, C-3), 72.12 (–, C-3 Glc), 71.03 (–, C-2 Glc), 69.95 (–, C-4 Glc), 67.79 (–, C-5 Glc), 63.66 (+, C-6 Glc), 62.59 (+, $CH_2N^+CH_2$), 56.84 (–, C-14), 56.26 (–, C-17), 50.83 (–, C-9), 50.00 (–, N^+CH_3), 42.46 (+, C-13), 40.87 (+, C-12), 39.69 (+, C-24), 38.95 (+, C-4), 37.20 (+, C-1), 36.75 (+, C-10), 36.34 (+, C-22), 35.96 (–, C-20), 32.09 (+, C-7), 32.00 (–, C-8), 29.70 (+, C-2), 28.41 (+, C-16), 28.20 (–, C-25), 24.47 (+, C-15), 24.01 (+, C-23), 23.01 (–) and 22.75 (–, C-26, 27), 22.34 (–, C(O)CH_3), 21.23 (+, C-11), 20.92 (–) and 20.80 (–, 2 C(O)CH_3), 20.43 (+, $CH_2CH_2CH_2$), 19.50 (–, C-19), 18.89 (–, C-21), 12.03 (–, C-18); $C_{45}H_{74}INO_8$ (883.98, 883.4459), HRESIMS: found 756.5395, calcd for $[M-I]^+$ 756.5409.

3.9. General procedure for the removal of acetyl groups

Freshly prepared sodium methylate (0.1 N, 1.4 mL) in MeOH was added to the protected glucosides **5a–d** (0.141 mmol) in MeOH (3.5 mL). The reaction mixture was kept at 20 °C until no starting material was detectable (TLC) and then treated with Dowex 50w × 8 (H^+) ion-exchange resin. The resin was filtered off, washed with MeOH, the combined filtrate was evaporated and the crude product was purified by the FC on RP-18 silica gel Lichroprep® (Merck). The respective starting glucosides, reaction time, chromatographic eluent, yield, and the physical and spectroscopic data for **7a–d** are as follows.

3.9.1. Cholest-5-en-3β-yl 6-deoxy-6-pyridinio-β-D-glucopyranoside methanesulfonate (7a)

Compound **5a** (0.117 g, 0.141 mmol), 1 h, 10:100:1 $CHCl_3$–MeOH–water; **7a** as a beige amorphous solid (0.078 g, 78%); $[\alpha]_D^{25}$ −14.2 (*c* 1.0, $CHCl_3$–CH_3OH, 2:1); ^{1}H NMR (400 MHz, 2:1 $CDCl_3$–CD_3OD): δ 8.87 (m, 2H), 8.50 (m, 1H) and 8.01 (m, 2H, $C_5H_5N^+$), 5.17–5.21 (m, 1H, H-6), 4.94 (dd, 1H, $J_{6b,5}$ 3.0, $J_{6b,6a}$ 13.5, H_b-6 Glc), 4.69 (dd, 1H, $J_{6a,5}$ 7.6, $J_{6a,6b}$ 13.5, H_a-6 Glc), 4.26 (d, 1 H, $J_{1,2}$ 7.8, H-1 Glc), 3.66–3.73 (m, 1H, H-5 Glc), 3.39 (br t, 1H, $J_{3,2}$, $J_{3,4}$ 9.0, H-3 Glc), 3.15–3.25 (m, 1H, H-3), 3.00–3.11 (m, 2H, H-4 Glc, H-2 Glc), 2.66 (s, 3H, SCH_3), 2.07–2.22 (m, 2H, H-$4_{a,b}$), 1.87–1.98 (m, 3H, H_b-7, $H_{a,b}$-12), 1.66–1.82 (m, 3H, H_b-1, H_b-2, H_b-16), 0.95–1.59 (m, 20H, H cholesterol), 0.90 (s, 3H, H-19), 0.84 (d, 3H, $J_{21,20}$ 6.5, CH_3-21), 0.78 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6, CH_3-26, 27), 0.60 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz): δ 145.84, 145.71, 139.98, 127.91, 122.13, 101.74, 79.73, 75.84, 73.67, 73.30, 70.91, 62.51, 56.73, 56.19, 50.21, 42.26, 39.73, 39.43, 38.81, 38.48, 37.15, 36.58, 36.11, 35.70, 31.80, 29.60, 28.10, 27.88, 24.14, 23.73, 22.49, 22.25, 21.01, 19.05, 18.51, 11.65; $C_{39}H_{63}NO_6S$ (705.99, 705.4274), MALDI-TOFMS: *m/z* 610.492 $[M-MsO]^+$; HRESIMS: found 610.4472, calcd for $[M-MsO]^+$ 610.4471.

3.9.2. Cholest-5-en-3β-yl 6-deoxy-6-(*N*-methylimidazolio)-β-D-glucopyranoside methanesulfonate (7b)

Compound **5b** (0.095 g, 0.11 mmol), 3 h, 100:1 MeOH–water; **7b** as a beige amorphous solid (0.037 g, 45%); $[\alpha]_D^{25}$ −44 (*c* 1, 1:2 $CHCl_3$–MeOH); ^{1}H NMR (400 MHz, 2:1 $CDCl_3$–CD_3OD): δ 9.02 (s, 1H), 7.43 (s, 1H) and 7.45 (s, 1H, imidazole), 5.30–5.35 (m, 1 H, H-6), 4.39–4.55 (m, 3H, H-1 Glc, $H_{a,b}$-6 Glc), 3.94 (s, 3H, CH_3N^+), 3.57–3.64 (m, 1H, H-5 Glc), 3.39–3.49 (m, 2H, H-3, H-3 Glc), 3.09–3.17 (m, 1H, H-4 Glc), 2.97–3.04 (m, 1H, H-2 Glc), 2.69 (s, 3H, SCH_3), 0.94–2.30 (m overlapped with s at 0.98, 28H, H cholesterol), 0.99 (s, 3H, CH_3-19), 0.89 (d, 3H, $J_{21,20}$ 6.5, CH_3-21), 0.84 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6, CH_3-26, 27), 0.66 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz): δ 139.82, 124.02, 123.51, 122.45, 101.86, 79.98,

76.22, 73.70, 73.58, 70.65, 57.07, 56.51, 50.73, 50.56, 42.61, 40.07, 39.77, 38.87, 37.58, 36.97, 36.45, 36.33, 36.03, 32.17, 29.93, 28.43, 28.21, 24.49, 24.06, 22.84, 22.60, 21.35, 19.42, 18.86, 11.99; $C_{38}H_{64}N_2O_8S$ (708.99, 708.4383), MALDI-TOFMS: *m/z* 613.111 $[M-MsO]^+$; HRESIMS: found 613.4580, calcd for $[M-MsO]^+$ 613.4581.

3.9.3. Cholest-5-en-3β-yl 6-deoxy-6-(*N*-methylmorpholinio)-β-D-glucopyranoside iodide (7c)

Compound **5c** (0.102 g, 0.115 mmol), 3 h, 100:1 MeOH–water; **7c** as a slightly yellow amorphous solid (0.045 g, 52%); $[\alpha]_D^{25}$ −31 (*c* 1, 1:2 $CHCl_3$–MeOH); ^1H NMR (400 MHz, Py-d_5): δ 5.07–5.19 (m, 2H, H-6, H-1 Glc), 4.58–4.66 (m, 1H, H-5 Glc), 4.45–4.52 (m, 1H, H_b-6 Glc), 3.95–4.26 (m, 1H, H-3 Glc), 3.73–4.14 (m, 9H, CH_2OCH_2, $CH_2N^+CH_2$, H_a-6 Glc), 3.56–3.72 (m, 6H, H-3, H-2 Glc, H-4 Glc, CH_3N^+), 2.52–2.61 (m, 1H, H_b-4), 2.15–2.28 (m, 1H, H_a-4), 0.76–1.90 (m, 28H, H cholesterol), 0.73 (d, 3 H, $J_{21,20}$ 6.5, CH_3-21), 0.69 (s, 3H, CH_3-19), 0.64 (d, 6 H, $J_{26,25}$, $J_{27,25}$ 6.6, CH_3-26, 27), 0.41 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz): δ 140.37, 122.53, 100.66, 79.57, 75.71, 73.37, 71.30, 69.94, 66.07, 65.13, 61.43, 60.84, 57.01, 56.44, 50.45, 50.30, 42.51, 39.98, 39.71, 38.76, 37.54, 36.94, 36.40, 35.96, 32.11, 29.82, 28.39, 28.18, 24.45, 24.03, 22.88, 22.64, 21.27, 19.40, 18.86, 15.16, 12.00; $C_{38}H_{66}INO_6$ (759.85, 759.3935), MALDI-TOFMS: *m/z* 632.161 $[M-I]^+$; HRESIMS: found 632.4891, calcd for $[M-I]^+$ 632.4890.

3.9.4. 5-Cholest-5-en-3β-yl 6-deoxy-6-(*N*-methylpiperidinio)-β-D-glucopyranoside iodide (7d)

Compound **5d** (0.041 g, 0.046 mmol), 3 h, 10:100:1 $CHCl_3$–MeOH–water→4:10 chloroform–methanol; **7d** as an amorphous solid (0.032 g, 89%); $[\alpha]_D^{25}$ −24 (*c* 1, 1:2 $CHCl_3$–MeOH); ^1H NMR (400 MHz, $CDCl_3$–CD_3OD, 4:1) δ 5.26–5.31 (m, 1H, H-6), 4.45 (d, 1 H, $J_{1,2}$ 7.8, H-1 Glc), 3.77–3.85 (m, 1H, H-5 Glc), 3.67–3.74 (m, 1H, H_b-6 Glc), 3.28–3.49 (m, 10H, H-3, CH_3N^+, $CH_2N^+CH_2$, H-3 Glc, H_a-6 Glc), 3.05–3.18 (m, 2H, H-2 Glc, H-4 Glc), 2.17–2.38 (m, 2H, $H_{a,b}$-4), 1.79–2.02 (m, 9H, cholesterol, $CH_2CH_2CH_2$), 0.95–1.84 (m, 26H, H cholesterol, $CH_2CH_2CH_2$), 0.94 (s, 3H, CH_3-19), 0.84 (d, 3H, $J_{21,20}$ 6.5, CH_3-21), 0.79 (d, 6H, $J_{26,25}$, $J_{27,25}$ 6.6, CH_3-26,27), 0.61 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz): 139.73, 123.09, 100.56, 79.71, 75.15, 73.20, 71.03, 69.92, 63.84, 62.36, 56.98, 56.38, 50.76, 50.28, 42.50, 40.07, 39.71, 38.85, 37.36, 36.81, 36.34, 35.92, 32.14, 32.00, 29.74, 28.36, 28.18, 24.45, 24.06, 22.89, 22.68, 21.23, 20.49, 19.45, 18.90, 11.98; $C_{39}H_{68}INO_5$ (757.88, 757.4142), MALDI-TOF MS: *m/z* 630.174 $[M-I]^+$.

3.10. Lipid formulations

Solns of cationic glycolipids **7a**–**d** in Me_2SO were prepared as follows: the weighed samples of the lipids were supplemented with Me_2SO, and the mixtures were intensively vortexed and sonicated in an ultrasonic bath (Cole-Parmer, USA) until soln clarification. The stock solns of the lipids in Me_2SO (1 mM) were stored at −20 °C.

For producing the liposomes, lipids **7a**–**d** were supplemented with the DOPE soln in $CHCl_3$ (Fluka) and the solvent was carefully removed under an argon flow. The obtained lipid film was dried under a vacuum (0.1 Torr) for 1 h. Water was added, and the lipid film was hydrated at 50 °C by intensive vortexing. The samples were flushed with argon and sonicated for 20 min in a bath-type sonicator. Liposomes were stored at 4 °C under argon atmosphere.

3.11. Oligonucleotide

The 25-mer oligodeoxyribonucleotide with an aminohexyl linker at the 3'-end (5'-TAC AGT GGA ATT GTA TGC CTA TTA T-3') was synthesized by a phosphoramidite method and purified by HPLC (produced at the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Russia). The purity of oligonucleotide, as analyzed by electrophoresis in 20% PAAm/8 M urea gel, was 95–98%. FITC-labeling of the oligonucleotide was performed as previously described.[54] FITC-ODN was isolated by conventional HPLC (Alliance, Waters, USA) using a Waters XTerra column and a linear acetonitrile concentration gradient. The purity of FITC-ODN samples as analyzed by electrophoresis in 20% PAAm/8 M urea gel was 95–98%. Concentrations of oligonucleotides were measured in a BioMate 3 (Termo Electron Corporation, USA) spectrophotometer. FITC-ODN soln was stored at −20 °C until used.

3.12. siRNA duplex

The siRNA targeted to EGFP mRNA (encoding enhanced green fluorescent protein) with the following sense and antisense strand sequences was used: 5'-GAA CGG CAU CAA GGU GAA CTT-3' (sense) and 5'-GUU CAC CUU GAU GCC GUU CTT-3' (antisense).[55] The chosen siRNA-EGFP was able to inhibit the EGFP expression in transgenic cells. The sense and the antisense strands of siRNA synthesized by the solid-phase phosphoramidite method were kindly provided by Dr. Venyaminova (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Russia). To obtain the siRNA duplexes, equal amounts of oligoribonucleotides were suspended in hybridization buffer (50 mM potassium acetate, 1 mM magnesium acetate, and 15 mM HEPES/KOH pH 7.4) and annealed by heating to 90 °C for 5 min with subsequent slow cooling. The resulting siRNA duplexes were analyzed by electrophoresis in 15% PAAm/8 M urea gel. The stock solutions were stored at −20 °C.

3.13. Preparation of the complexes of cationic glycolipids (or liposomes) and nucleic acids

Prior to use, the complexes of cationic glycolipids (or liposomes) and nucleic acids were formed in a serum-free Opti-MEM medium (Invitrogen, USA) by vigorously mixing 25 μL of glycolipid or liposomal solution and 25 μL of oligonucleotide or siRNA taken at an appropriate concentration; the resulting mixtures were incubated for 20 min at a room temperature.

3.14. Biological methods

3.14.1. Cell line and cultivation conditions

The genetically modified BHK IR780 cell line containing constantly expressed EGFP gene insert in the genomic DNA, obtained from the parental cell line of BHK (hamster kidney) cells, was kindly provided by Prof. Prasolov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia). The initial BHK and the transgenic BHK IR780 cell lines were maintained in the DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin in a humidified atmosphere containing 5% CO_2/95% air at 37 °C and were regularly passaged to keep the exponential growth.

3.14.2. Cell viability test (MTT assay)

The cytotoxicities of glycolipids and liposome formulations were assessed using a colorimetric assay based on the reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by living cells.[44] Prior to the cytotoxicity assay, the conditions for cells growth were optimized to achieve 90% of cell monolayer after 48 h incubation. The cells were seeded into 96-well plates (10^4 cells per well) and cultivated in 100 μL of the DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere

with 5% CO_2 for 24 h to adhere. Then the medium in wells was replaced with 150 μL of DMEM without antibiotics (with or without 10% FBS) containing different concentrations of the cationic glycolipids or liposome formulations (1–120 μM). After 24 h incubation in the presence of glycolipids or liposomes, MTT was added to the cells to a final concentration of 0.5 μg/mL. The optical density of the samples was measured 3 h after MTT addition at 570 nm in a Multiscan RC (Labsystems). All experimental points were run in four replicates. The results were represented as a percentage of living cells versus the control (untreated cells, 100%). S.D. was below 5%. For each glycolipid (liposome formulation), the IC_{50} value, that is, the concentration of a compound providing for 50% cell lethality was determined.

3.14.3. Cellular delivery of FITC-labeled oligonucleotide and siRNA

The delivery efficiency for the nucleic acids complexed with glycolipids was evaluated in transfection experiments with the 25-mer FITC-labeled oligonucleotide and anti-EGFP siRNA.

For FITC-ODN transfection experiments, BHK cells were seeded into 24-well plates (3×10^4cells/well) in the DMEM containing 10% FBS and antibiotics 24 h before transfection and grown in an incubator at 3 °C in the presence of 5% CO_2. Immediately before transfection, the cells were rinsed with PBS supplemented with 200 μL of fresh serum-free culture medium or the medium containing 10% FBS. The complexes of cationic lipids (or liposomes) (10 μM or 20 μM) and FITC-ODN (0.8 or 5 μM) were formed in 50 μL of serum-free Opti-MEM medium as described above. Cells were supplemented with these complexes and incubated for 4 h. FITC-ODN delivery with Lipofectamine2000 was done according to manufacture protocol (www.invitrogen.com).

For siRNA delivery, BHK IR780 cells were seeded into 24-well plates (2×10^4 cells/well) in the DMEM medium containing 10% FBS and antibiotics 24 h before transfection and grown in an incubator at 37 °C in the presence of 5% CO_2. Immediately before transfection, the cells were rinsed with PBS and supplemented with 200 μL of fresh serum-free culture medium or the medium containing 10% FBS. The complexes of siRNA and cationic glycolipids (liposomes) were preformed using 50 nM siRNA and glycolipids at concentrations of 5, 10, 20, or 40 μM as described above and were added to the cells; 4 h after transfection, the culture medium was replaced with the DMEM containing 10% FBS. siRNA delivery with Lipofectamine2000 was done according to manufacture protocol (www.invitrogen.com).

3.14.4. FACS analysis

Flow cytometry studies were used to characterize the efficiency of cell transfection with FITC-labeled oligonucleotide and the EGFP-targeted siRNA mediated by glycolipids (liposome formulations). In the case of oligonucleotide delivery, the cells were analyzed immediately after transfection (4 h after addition of glycolipid/oligonucleotide complexes). In the case of siRNA delivery, cells were analyzed 72 h after transfection (this time is necessary for the already synthesized protein to be degraded, because the half-life time for EGFP is considered to be 48 h). The cells were rinsed twice with PBS and detached from the plate by trypsin treatment (0.5 mg/mL in PBS) at 37 °C for 2 min. The trypsinized cells were resuspended in the culture medium and collected by centrifugation (a Contron T42K centrifuge, Centricon Instruments) at 1000 rpm for 10 min at 4 °C. After centrifugation, the medium was removed and the cells were washed with PBS and fixed with 2% formaldehyde in PBS. The resulting samples assayed by cytofluorometry using a fluorescence-activated cell sorter (BD FACSAria, Becton Dickinson). Fluorescence intensity of individual cells was measured in relative fluorescence units (RFU). The fluorescence was collected from 3×10^4 individual cells. The percentage of FITC-positive cells was calculated by determining their fluorescence emission at a PMT equipped with a 502 LP dichroic mirror and a wavelength optical filter 530/30 nm. All experimental points were run in duplicate for statistical analysis; the standard deviation did not exceed 5%.

3.15. Physicochemical characterizations of liposomes and lipoplexes

Particle size and ζ potential were measured using a Delsa Nano dynamic light scattering instrument with ζ-sizing capacity (Beckman Coulter). For measuring complexes characteristics ODN in 450 mL of distilled water was mixed with an equal vol of liposomal formulation (in distilled water) at the ratio 0.8:20 mol and incubated for 15 min at room temperature.

3.15.1. Transmission electron microscopy: characterization of liposomes and ODN/liposome complexes by negative staining

The liposomal formulations or their complexes with ODN were prepared as described above. The samples were absorbed on copper grids covered with formvar film during 20 s, then dried by touch with filter paper. For contrasting, the grids were put on a drop of 2% phosphotungstic acid (pH 7.2) for 15 s. Excess stain was removed by filter paper. The grids were examined in Jem 1400 transmission electron microscope (JEOL, Japan) at 80 kV accelerating voltage. Digital images were collected by Veleta CCD camera (Olympus SIS, Germany).

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